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Preparation of Recombinant Proteins in Milk

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Summary

Using transgenic animals as the source of recombinant proteins has several specific advantages. Large amounts of proteins can be obtained, essentially from milk. These proteins are often properly processed. They are in a number of cases correctly folded, assembled, cleaved, glycosylated, γ-carboxylated, and so on. Purification of recombinant proteins from milk is not a particularly difficult task. The level of expression of foreign genes in milk cannot be predicted in all cases and appropriate vectors must be used. Generation of transgenic mice is popular but their production is quite limited. Transgenesis in larger animals, rabbits and farm animals, is achieved essentially by a few companies. Some recombinant proteins may be found in blood circulation and alter animal health. Milk from transgenic animals has become a quite attractive alternative to other sources of recombinant proteins.

Key Words: Recombinant proteins; milk; expression vectors; transgenic animals; post-translational modifications; purification.

1. Introduction

The preparation of recombinant proteins is currently achieved for different purposes. Researchers need proteins to study their biological activity and their structure using native molecules and mutants obtained by genetic engineering. In a number of cases, this approach is essential to designing new drugs interacting with proteins and potentially utilizable as pharmaceuticals.

For centuries, our ancestors have used plant extracts as pharmaceuticals. Proteins remained out of the game until their major roles in living organisms were identified. For decades a small number of proteins were used as pharmaceuticals. Only a few naturally abundant proteins that can be extruded from human or animal organs or blood were

From: *Methods in Molecular Biology, vol. 267:*
Recombinant Gene Expression: Reviews and Protocols, Second Edition
Edited by: P. Balbás and A. Lorence © Humana Press Inc., Totowa, NJ

Applicants: Hermona Soreq et al.
Serial No. : 09/310,638
Filed: May 12, 1999
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used. Insulin, human growth hormone, and blood-clotting factors are good examples of this approach. Genetic engineering rapidly offered quite new possibilities. Human insulin and growth hormone are now prepared from recombinant bacteria. A number of proteins, however, are too complex to be prepared from recombinant bacteria or yeast. Animal cells are needed to proceed to the numerous and subtle posttranslational modifications that are required to obtain active stable and nonimmunogenic proteins. Cultured animal cells are currently used to prepare some recombinant proteins. Recombinant Chinese hamster ovary (CHO) cells are presently the only source of several recombinant proteins. The idea of using transgenic animals as bioreactors was first suggested in 1982. Several biological fluids from animals can be used as the source of recombinant proteins (*1*). Milk is probably the most attractive. It is the system which is presently the closest to industrial application.

Using transgenic animals as bioreactors raises specific problems. The first is to construct vectors leading to high expression of the recombinant proteins. A second problem is to generate, breed, and milk animals. The purification of the recombinant protein from milk is the next step, which is not particularly difficult but requires strict protocols if recombinant proteins are to be used as pharmaceuticals.

This chapter describes the main steps of recombinant protein preparation from milk of different animals.

2. Materials and Methods

2.1. Gene Constructions

Targeting recombinant proteins to milk implies that a milk protein promoter is fused to the coding sequence of the protein of interest. The design of the expression vectors is discussed below. Vectors may be conventional and contain a few regulatory elements.

Alternatively, long genomic DNA fragments may be used to optimize transgene expression. The conventional methods of genetic engineering are to be used to reach this goal.

2.2. Design of Expression Vectors

Expressing a transgene in an appropriate manner is not an easy task. The first experiments carried out in the early 1980s revealed that a gene construct that is quite active in transfected cells may be only weakly expressed in transgenic animals. Moreover, each line of animals shows a specific pattern of transgene expression. In addition, a number of transgenes remain silent. This is a clear limitation for the use of transgenic animals. In a majority of cases, however, researchers prepare an exceeding number of transgenic founders until they find the lines expressing the foreign gene as they expected. This approach implies a wastage of time and animals but it appears sufficient to obtain 1000–10,000 copies of recombinant protein per cell exhibiting the expected biological action. This relative but real success of the expression vectors dissuaded researchers for years from improving them.

The situation is different when recombinant proteins are to be prepared in milk. Highly efficient expression vectors are then needed to obtain the expected large

amounts of proteins. Costly transgenic farm animals must often be used and reliable expression vectors are highly preferable. On the other hand, the recombinant proteins must be restricted to milk as much as possible to avoid any potential deleterious effects on animals. The groups involved in recombinant proteins in milk and a few others have done systematic studies to improve expression vectors. Designing the ideal vector for transgene expression remains an impossible task. Yet basic studies on the mechanisms of gene expression and empirical observations have led to defined rules for vector construction and to identification of pitfalls that can be avoided.

A functional transcription unit is composed of several elements that cooperate with each other. These elements and their mode of action are not all known. A gene construction consists of associating more or less known elements. This may generate nonfunctional vectors for unknown reasons. The major recommendations for designing reliable vectors have been described elsewhere (*2*). They may be summarized as follows.

2.2.1. Promoters

The promoter region contains the DNA sequences that allow the formation of the transcription initiation complex and, in most cases, those that give the specificity of expression. Promoters are restricted to the 100–200 bp preceding the transcription start site. Enhancers have a global amplification effect. It is now admitted that they do not increase promoter potency by enhancing the chance of having active transcription complex. Enhancers may reinforce tissue specificity of expression. In most cases, they are found in the 0.5–10 kb region preceding the promoter. Enhancers may be found in the transcribed region and after the transcription terminator, and they can be added to a construct in the region upstream of the promoter but also in the transcribed region, even within an intron (*3*).

An increasing number of promoters are becoming available. They may be chosen using published data to drive a cell specific expression in vivo.

2.2.2. Insulators

Numerous experiments have shown that a transgene is generally poorly expressed (1) when it contains a cDNA rather than the corresponding genomic DNA; (2) when it is integrated as multiple copies; (3) when it contains nonmammalian DNA sequences, namely CpG-rich sequences; and (4) when it is integrated in a silent region of chromatin. The last phenomenon is known as "position effect." Vector constructions may be performed taking these observations into account.

Studies carried out during the 1990s contributed to establishing the concept of insulators (*see* Chapters 28 and 29). It is now acknowledged that groups of genes, acting or not acting in a coordinated manner, are bordered by DNA sequences that insulate them from the rest of the chromosome. These sequences have been named insulators or LCRs (locus control regions) (*4*). Insulators are composed of multiple elements. Some are silencers, which block the action of the enhancers present in the vicinity of the integrated transgene. Other elements are considered as chromatin openers (*5*), which bind protein complexes inducing histone acetylation. This is accompanied by a local histone

and DNA demethylation (*6*). Insulators also often contain AT-rich matrix-attached regions (MAR).

The addition of insulators to gene constructs greatly enhances transgene expression. A higher number of lines express the transgene and the mean level of expression is also enhanced. The addition of the 5'HS4 element from the LCR of the chicken β-globin locus—preferably on both sides, but also only upstream of the promoter—allowed cDNAs driven by the human EF1α gene (*7*) or the rabbit WAP (whey acidic protein) gene promoter (*8*) to be expressed in all rabbit and mouse lines, respectively.

Several long genomic DNA fragments allow their genes to be expressed at a high levels (corresponding to the known potency of the promoters) in a tissue-specific manner and as a function of the integrated copy number (*5*). This was also observed for two milk protein genes, human and goat α-lactalbumin gene (*9,10*) and pig WAP gene (*11*). This long genomic cDNA fragment can be used to express foreign genes with high efficiency (*12*). In the future, identified insulator elements might be used instead of the whole BAC to construct shorter vectors, which are easier to manipulate.

2.2.3. Transcribed Regions

The transcribed regions of the genes contain a number of regulatory elements that control exon splicing, mRNA transfer to cytoplasm translation, mRNA targeting, and mRNA half-life.

A vector for transgene expression must contain a least one intron, which is required for the transfer of the mRNA to cytoplasm, although several introns may be present. In this respect, minigenes are generally more efficient than vectors containing a single exogenous intron. This may be due to multiple factors, including the presence of transcription enhancers within introns.

The intron may be added before or after the cDNA. However, the presence of the intron before the cDNA, rather than after, prevents the nonsense-mediated decay (NMD) mechanism to inactivate the mRNA. This destruction mechanism is considered as a quality control system, which eliminates all the mRNA that has a premature terminator codon located more than 50 nucleotides from the last acceptor site of an intron (*13*). This situation is never found in natural mRNA. When it happens it is recognized by cells at the result of a mutation generating a terminator codon. An appropriate construction may avoid the NMD mechanism to inactivate a mRNA when an intron is added after the cDNA.

Alternative splicing is a quite frequent event in higher-vertebrate mRNA. It is a way to control gene expression and to enhance the diversity of the cellular mechanisms using a limited number of genes. The association of introns and cDNAs often leads to alternative splicing, which prevents the transgene from driving the synthesis of the expected protein. *In silico*, studies may predict the combination of donor and acceptor splicing sites. However, the splicing of exon is a complex phenomenon implying a number of cellular proteins that are present in some cell types only and induced by multiple cellular events. Hence, the use of splicing sites cannot be predicted only *in silico*. An evaluation of the construct may be done using transfection into cultured cells. The

mouse mammary cell line HC11 may be helpful for this purpose. One way to avoid inappropriate splicing is to mutate the splicing sites of the construct.

2.2.4. Nontranslated Regions (UTR)

The 5'UTR may contain sequences that control translation. The elements known as internal ribosome entry sites (IRES) seem to play this role (*14*). Selected 5'UTR may be added before the cDNAs to favor their translation.

The 3'UTR often contain elements that control mRNA stability and mRNA targeting (*3*). AU-rich regions are known to be potent destabilizers of mRNAs. On the contrary, the motif C/UCCANXCCCU/A PyX.UCC/UCC stabilizes various mRNAs.

2.2.5. Codon Optimization

Each group of living organism uses isocodons preferentially. Using mammalian codons, and preferably those found in the mRNA of milk protein genes, may greatly increase transgene expression by favoring translation and stability of its mRNA.

2.2.6. Protein Secretion

To be found in milk, a protein must contain a signal peptide. Naturally secreted proteins have such a signal. Cellular proteins may be secreted with good efficiency in many cases when a sequence coding for signal peptide is added to the cDNA. However, the protein may contain sequences targeting it to a cellular compartment other than the endoplasmic reticulum. This may create a conflict and reduce the secretion rate of the protein.

2.2.7. Vectors for the Simultaneous Expression of Two Coding Sequences

A number of proteins are composed of several subunits that must be coexpressed and assembled in the Golgi apparatus.

The coinjection of two or three independent gene constructs leads to a cointegration and coexpression of the different coding sequences. This has been achieved successfully with different constructs and in several tissues of mammals. This approach is expected to be less efficient with long vectors such as BAC, which are often integrated as a single copy.

Another possibility is to associate the two constructs by ligating them in vitro before microinjection. This may be difficult when each construct is long. On the other hand, the vicinity of the two constructs may induce a phenomenon known as transcription interference, which may reduce expression of both genes.

A third approach may consist of using IRES. These sequences are known to allow the expression of the second cDNAs of bicistronic constructs. Numerous IRESs have been identified and are available. Their mechanism of action is still not well understood (*14*). A fact that has been regularly observed is that the IRESs commonly used allow the simultaneous expression of two cDNAs of bicistronic mRNAs but at a level that is

lower or much lower than this observed with each cDNA used alone. Hence, IRESs are appropriate to express cDNAs at a low level but perhaps not to produce recombinant proteins in milk at a sufficient rate.

A systematic study revealed that the size of the region between the terminator codon and IRES should be about 80 nucleotides to optimize expression of both cDNAs of bicistronic mRNAs (*14*).

2.3. DNA Preparation for Microinjection

The quality of DNA is essential to obtain transgene animals with good chance of success. Plasmid sequences must be eliminated because they may considerably reduce transgene expression. Inserts containing only the elements of expression vectors must be separated from the plasmid in agarose gel and purified. The conventional inserts (not exceeding 30 kb) may be purified from agarose by classical methods; Gene Clean (Bio101) is one of them. Centrifugation at 10,000*g* for at least 30 min is then required to eliminate particles that may block DNA flow in micropipets. Alternatively, filtration using 0.22-μ filters can be achieved.

For long fragments (50–500 kb) contained in bacterial artificial chromosomes (BAC) or yeast artificial chromosomes (YAC), the inserts must be released using agarose and purified by dialysis (*11*). The presence of polyamines is required to protect the longer fragments from shearing (*15*).

2.4. Generation of Transgenic Animals

Microinjection of DNA into embryo pronuclei remains the most frequently used method to generate transgenic mammals. The protocol to generate transgenic mice has been described in several books. The readers may refer to two of them (*16,17*). The preparation of larger transgenic mammals by DNA microinjection requires more specific methods, which have been described earlier (*18*).

Alternative methods to generate transgenic animals can be implemented. Transposons and retroviral vectors may be used in mice. Gene transfer into somatic cells further used as nucleus donors for cloning is presently the most powerful method to generate transgenic ruminants (*19*).

2.5. Purification of Recombinant Proteins From Milk

Milk collection from ruminants is achieved using conventional milking machines. In mice, rabbits, and pigs, the mammary gland has no cisternae. Milk stored in the mammary gland is ejected by an active process. Oxytocin (up to 5 IU) must be injected intraperitoneally in mice. Up to 1 mL of milk may be obtained by applying mild vacuum to the teats. Up to 100 mL milk can be obtained per day from lactating rabbits using essentially the same protocol. Alternatively, the mammary gland of mice (or rabbits) separated from their pups for one day may be collected and kept for a few hours in a Petri dish on ice. Quantitative milk collection is obtained in this way (up to 1 mL from mice and 200 mL from rabbits). Although this protocol implies the sacrifice of the animals (*20*), it is appropriate to obtain easily limited amounts of milk.

Recombinant proteins are found in the lactoserum, which is the milk fraction remaining after the elimination of lipids and caseins. Lipids are removed after centrifugation. Casein micelles can be eliminated by different methods, including high-speed centrifugation, specific precipitation at pH 4.6, calcium excess, or polyethyleneglycol (*21*). Available chromatographic methods may then be used to purify the proteins of interest from the lactoserum.

3. Conclusions

The major reason that justifies the use of milk as a source of recombinant proteins is the production of pharmaceuticals. Indeed, for limited production, CHO cells in fermentor may be sufficient. However, this technique is not so easy to manage and it may not be implemented by an academic laboratory. The use of transgenic mice may appear easier.

Only a few proteins—mainly monoclonal antibodies—are candidates to become pharmaceuticals, although their number is rapidly growing. Yet, many proteins are needed for experimenters to study their properties, including their potential clinical use. For very small amounts of proteins (not exceeding 10–100 mg), transgenic mice may be used. Their milk starts being available 4 mo after DNA microinjection into embryos. For larger amounts, rabbits are quite appropriate. The lactating animals start being available 6–7 mo after DNA microinjection, and one female can provide up to 2 L of milk over a period of 1 mo (corresponding to one lactation).

One month later, the female is again lactating. Hence, rabbits can provide researchers with recombinant proteins in sufficient amounts to study their properties in depth. Rabbits may reproduce at a high rate and herds are rapidly available. Hundreds of rabbits may be bred to produce 1–10 kg of recombinant proteins per year.

For higher amounts of proteins, goats, sheep, cows, and potentially pigs must be used. This is justified only for the production of pharmaceuticals or proteins, such as spider silk, having specific properties.

The methods to be implemented tend to be standardized. The expression vectors have been and are still being improved. One of them, known as pBC1 (Invitrogen), is sufficient in most cases to produce small quantity of proteins in mouse milk. Other vectors are available in laboratories studying milk protein gene promoters. The available efficient promoters are from the following genes: ruminant αS1- and β-caseins, rabbit and mouse WAP, and ovine β-lactoglobulin. All these promoters are patented by the companies that use them.

The techniques to generate transgenic mammals are standardized, even if they are still being improved.

One major problem of recombinant proteins is their posttranslational modifications (*22*). The available information indicates that the recombinant proteins found in milk may be not fully sialylated (*22*). Yet the proteins produced in milk may be slightly different from those secreted by CHO cells, but of similar quality. This was seen with human EC-superoxide dismutase prepared in CHO cells and in rabbit milk (*23*).

The incomplete glycosylation, cleavage γ-carboxylation, is clearly due to the lack of the appropriate enzymes in the mammary cells (*22*). A higher production of a recombinant

protein may lead to a lower achievement of posttranslational modifications. This is clearly due to a saturation of the enzymatic systems.

This shortage may be theoretically compensated by the overexpression of enzymes after transfer of the corresponding genes to embryos. It is interesting to note that the overexpression of the furin gene allowed human protein C in mouse milk to be almost quantitatively cleaved and transformed into an active protein (*22*).

The major posttranslational modifications to optimize are the following: sialylation, fucosylation, N-acetylglycosylation of the constant part of antibodies to stimulate killer lymphocytes, the addition of sialic acid as N-acetylneuraminic acid rather than *N*-glycosylneuraminic acid, which is not present in human proteins, and cleavage by furin (*24*).

The mammary cell proved to be able to assemble quite different protein subunits, such as fibrinogen, collagen, and antibodies (*25*). EC-superoxide dismutase is also an interesting case. This protein is a glycosylated homotetramer containing one copper ion per monomer. This protein was found at the concentration of several grams per liter in rabbit milk in a quite functional state (*23*); thus the mammary gland was capable of collecting large amount of copper from blood and adding it to EC-superoxide dismutase. Hence, the capacity of the mammary gland to associate proteins is probably not limiting. This may reflect its high capacity to assemble caseins to form micelles.

The implementation of transgenic animals to prepare recombinant proteins currently seems inevitable. This is clearly due to the specific advantages of this production system. It should be recalled that two of its major advantages are certainly its robustness and flexibility. Indeed, transgenic animals are not fragile in comparison to cells in a fermentor. They can be stored as living animals, as frozen embryos, and as sperm as well. The scaling up of a large fermentor is a long and costly process in comparison to the multiplication of animals.

To allow safe production of pharmaceuticals, the transgenic animals must be bred in narrowly controlled conditions. The points to consider defined by the US FDA and EU EMEA are not dissuading for the companies that develop this process (*26*).

Some recombinant proteins that are present in blood at a low concentration and not only in milk may alter the health of the animals. This was the case for human erythropoietin (*27*). In other cases, such as the production of EC-superoxide dismutase or of human protein C, the development of the mammary gland may be impaired, for unknown reasons.

Despite these minor drawbacks, the implementation of milk for the production of recombinant proteins appear inevitable. Indeed, all the available production systems, even if they are fully utilized, are expected to be unable to prevent a shortage of the overall production of recombinant proteins in this decade (*28,29*).

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